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<p>(54) Title: WOUND AND INJURY TREATMENT COMPOSITIONS AND THE USE THEREOF</p>		
<p>(57) Abstract</p> <p>The present invention relates to compositions and methods suitable for the treatment of wounds and for the promotion of wound healing. The invention also relates to methods for lessening restenosis of body lumens and to intraluminal stents and vascular biomaterials of grafts having anti-thrombosis and/or anti-restenosis properties. The present invention is particularly concerned with the treatment of endothelial cells although it may be used to treat other cells. The invention is based on the use of a combination of perlecan or active fragment thereof, and optionally a growth factor or active component thereof, and a source of cell adhesive protein. The source of cell adhesive protein may be plasma or serum.</p>		

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Wound and injury treatment compositions and the use thereof

Field of the Invention

The present invention relates to compositions and methods suitable
5 for the treatment of wounds and for the promotion of wound healing. The
invention also relates to methods for lessening restenosis of body lumens by
way of application of intraluminal stents, vascular biomaterials or grafts
having anti-thrombosis and/or anti-restenosis properties. The present
invention is particularly concerned with the treatment of endothelial cells
10 although it may be used to treat other cells.

Background of the Invention

Perlecan is the major heparan sulfate (HS) proteoglycan of basement
membranes and other connective tissues. The human molecule has a
protein core of molecular weight approximately 470,000, which has a well
15 described domain structure. Domain I is the N-terminal domain that carries
the 3 sites for heparan sulfate attachment. Domain II has homology to the
LDL receptor with strict conservation of the cysteine residues. Domain III
shares homology with the short arm of the laminin α chain. Domain IV is
the largest domain with a molecular weight in excess of 200,000, and has
20 immunoglobulin-like repeats similar to those found in the neural cell
adhesion molecule. Domain V is the C-terminal domain and has three
regions with homology to the globular domains of laminin α chain which
are interdispersed with four regions showing homology to epidermal growth
factor (Murdoch et al., 1992). These domains have been expressed in
25 bacterial cells and more recently in eucaryotic expression systems (Costell et
al., 1997; Groffen et al., 1996; Costell et al., 1996; Chakravarti et al., 1995;
Schulz et al., 1996). Recombinant domain III of mouse perlecan was shown
to be involved in the attachment of mammary tumour cells via an RGD
dependant pathway (Chakravarti et al., 1995). Domain I of mouse perlecan
30 expressed by transfected human kidney cells was present as a pool of

molecules, some of which had no glycosaminoglycan decoration and others which had either HS, chondroitin sulfate / dermatan sulfate (CS/DS) or a mix of both attached to them. The glycosaminoglycans chains that were coupled to the recombinant protein were approximately one-sixth the length of those
5 found on native perlecan (Costell et al., 1997). The study of the functional role of recombinant domain I, as well as those of the other domains is of intense interest.

Native perlecan has been purified from the mouse Englebroth Holm Swarm (EHS) tumour (Hassell et al., 1980), porcine kidneys (Gauer et
10 al., 1996), bovine kidneys (Hagen et al., 1993), human placenta (Isemura et al., 1987), from the conditioned medium of bovine aortic endothelial cells (Saku and Furthmayr, 1989), and from the extracellular matrix of cultured human fetal lung fibroblasts (Heremans et al., 1988; Aviezer et al., 1997). In most of these cases, the perlecan has been isolated using very stringent
15 chaotropic and denaturing agents such as guanidine and urea. In all cases the protein core isolated has had a molecular weight of approximately 400,000 for the mouse and around 450,000 for the human, however, the type and amount of glycosaminoglycan attached to this protein core has varied. In some cases, the protein core is decorated with HS only and on other
20 occasions it may be either unglycosylated or have CS/DS present (Iozzo et al., 1994; Hassell et al., 1980; Isemura et al., 1987). It is known that sequences in the protein core affect the amount and type of glycosaminoglycan which is attached, but the biological significance of the different glycosylation patterns remain unknown.

25. Bovine endothelial cells have been shown to attach to the protein core of native mouse perlecan via $\beta 1$ and $\beta 3$ integrins, however, if the HS is left on the protein core, the cells did not attach. The binding to the protein core was independent of the RGD sequence (which is present in domain III of mouse perlecan) and was inhibited by exogenous heparin (Hayashi et al.,
30 1992). Alternatively, other investigators have found that the attachment of

Rugli cells (a rat glioma derived cell line) to mouse perlecan did not involve the protein core and was totally dependant on the presence of the HS (Battaglia et al., 1993). Furthermore, the attachment of mesenchymal and epithelial derived tumour cell lines mirrored that of the Rugli cells.

5 However, cells derived from mouse endothelioma, human melanoma or human astrocytoma did not adhere at all, to the intact proteoglycan (Battaglia et al., 1993). These investigators concurred with the previous investigators that the binding was independent of the RGD sequence in mouse perlecan and that the $\beta 1$ integrin was involved (Battaglia et al., 1993)
10 but disagreed in that they found heparin had no effect on the adhesion of the glioma cells. An explanation for these somewhat divergent results would be that there are a number of alternative attachment mechanisms, each of which is used to different extents by different cell types. The attachment of cells to the protein core of human perlecan further supports
15 the hypothesis of alternative pathways being involved because the human homologue does not have the RGD sequence in domain III.

The binding and presentation of growth factors by perlecan is another important functional role of this matrix molecule. It has been shown that the heparin binding growth factor bFGF binds to the HS on
20 domain I of perlecan (Aviezer et al., 1994) and that it is released by biologically relevant enzymes, such as plasmin, collagenase and heparinases which may have a role in the regulation of the growth factor activity (Whitelock et al., 1996). Other heparin binding growth factors may also bind perlecan in a similar fashion.

25 Summary of the Invention

A major aim of a study which led to the present invention was to demonstrate the biological relevance of the bFGF/perlecan interaction in a vascular setting. This was achieved by studying the growth of endothelial and smooth muscle cells on a matrix of perlecan and fibronectin to which
30 had been attached bFGF. We have shown that vascular cells grow better on

a matrix that contains perlecan to bind and present the bFGF. If the perlecan was absent, the cells did not grow as well. Presumably because bFGF could not be made available to the cells. These data suggest a role for perlecan in the growth of vascular cells in the response to injury, and further
5 suggest that this proteoglycan may have a wider role in wound healing generally.

In a first aspect, the present invention provides a wound and injury treatment composition including perlecan or active fragment thereof, and a pharmaceutically acceptable carrier of diluent.

10 Preferably the composition of the first aspect further includes a heparin binding growth factor or active fragment thereof and/or a cell adhesive protein or an active fragment thereof.

Most preferably the composition of the first aspect further includes both a heparin binding growth factor or active fragment thereof and a cell
15 adhesive protein or an active fragment thereof

Preferably the perlecan is human perlecan or a biologically active fragment thereof.

The cell adhesive protein may be fibronectin or vitronectin or a combination thereof.

20 The heparin binding growth factor may be selected from one or more of the group consisting of basic fibroblast growth factor (bFGF), transforming growth factor α , transforming growth factor β , epidermal growth factor, platelet derived growth factor, heparin binding epithelial growth factor, granulocyte colony stimulating factor, vascular endothelial growth factor
25 (VEGF), VEGF-related growth factor, acidic FGF and other members of the FGF family.

Preferably the heparin binding growth factor is bFGF or VEGF.

The composition of the invention may include one or more other agents that promote the healing of wounds or treatment of symptoms

accompanying wounds. The other agent may be, for example, an anti-bacterial agent, anti-viral agent, anti-fungal agent or local anaesthetic.

The composition may be in the form of a liquid, semi-solid or solid.

5 The composition may be in the form providing controlled or sustained release. The carrier may be a biomaterial. The biomaterial may be formed in whole or in part from any suitable material, for example, polymers including biodegradable polymers.

The carrier may be a bandage. The carrier may be a catheter or the like, for example, an angioplastic catheter or other intraluminal catheter.

10 The active component(s) of the composition of the invention may be present in any suitable amount. The component(s) may be present in an amount of about 0.0001 to about 10% of the composition. Preferably, the active components are present in an amount of about 0.0001 to 1% of the composition.

15 In a second aspect, the present invention provides a method of treatment of an injury in a subject, the method including administering to the subject, perlecan or active fragment thereof.

20 Preferably the method of the second aspect includes administration of a heparin binding growth factor or active fragment thereof, and optionally a source of cell adhesive protein or active fragment thereof.

The heparin binding growth factor may be selected from the group set out above. Preferably the heparin binding growth factor is bFGF.

The source of cell adhesive protein may be fibronectin or vitronectin or a combination thereof.

25 The source of cell adhesive protein may be plasma or serum. Preferably the plasma or serum is of human origin. The plasma or serum may originate from the treated subject at the time of the procedure or injury.

The injury may be in the form of cellular damage such as that resulting from burns, sores, incisions or wounds.

The injury may be cellular injury or trauma occasioned by a surgical procedure. The cells may be vascular cells.

The present invention has particular application in the prevention or minimisation of restenosis. Restenosis is the reclosure of a peripheral or
5 coronary artery following trauma resulting from the opening of a stenosed portion of the artery by angioplasty procedures such as coronary angioplasty or by procedures such as bypass grafting, removal of material by atherectomy, coronary artery bypass surgery or insertion of stents. Restenosis is believed to result from an extension of the natural healing
10 reaction to the injury of the arterial wall that is caused by angioplasty procedures, or to result from localised tissue reaction to the foreign material in an implanted stent or synthetic vascular graft. The healing reaction begins with the thrombotic reaction at the site of the injury. The final result of the complex steps of the healing process can be the uncontrolled
15 migration and proliferation of medial smooth muscle cells, combined with their extracellular matrix production (ECM), until the artery or vascular graft is again stenosed or occluded.

In a third aspect, the present invention provides a method for inhibiting or reducing restenosis and/or thrombosis in a subject, the method
20 including treating the subject with perlecan or active fragment thereof.

The method of the third aspect may include treatment of the subject with heparin binding growth factor or active fragment thereof, and/or a source of cell adhesive protein or active fragment thereof.

Preferably, the method of the third aspect includes treatment of the
25 subject with a heparin binding growth factor or active fragment thereof, and optionally a source of cell adhesive protein or active fragment thereof.

The growth factor and source of cell adhesive protein may be that set out above. Preferably the growth factor is bFGF.

The method of the third aspect may involve treatment before, during
30 or following a surgical procedure such as coronary angioplasty, atherectomy, arterial bypass graft surgery, biomaterial implantation or the like.

One means of preventing restenosis in angioplasty procedures is by the use of metallic intravascular stents, which are permanently implanted in the coronary or peripheral vessel. The stent may be inserted by catheter into the vascular lumen and expanded into contact with the diseased portion of the arterial wall, thus providing a mechanical support of the lumen. Despite the high initial success rate, early and late complications such as thrombotic closure and restenosis are common.

The problems with the metallic stents have resulted in the development of new stents to produce a non-thrombogenic stent surface and avoid the problems of restenosis and neointimal hyperplasia. New designs have been developed relating to the composition of the stents and the geometry thereof, additionally, various subtle changes such as thickness of filaments, alloy composition ratio, surface roughness and biocompatible or therapeutic coatings have been investigated.

One example of such a development is to coat the surface of a metal stent with genetically engineered endothelial cells to decrease the thrombogenicity. Polymer coatings have also been employed to render the stent surface less thrombogenic. US patent 5,555,182 describes a method for preventing restenosis in a body lumen treated to open a luminal restriction by using a stent body coated with fibrin and heparin.

The present invention further provides a means by which the occurrence of restenosis and/or thrombosis following angioplasty procedures or the like may be prevented or lessened.

Accordingly in a fourth aspect, the present invention provides a method for preventing or reducing restenosis and/or thrombosis in a body lumen treated to open a luminal restriction, the method including the steps of providing a stent body, vascular graft implant or the like, treating the stent body, vascular graft or implant with perlecan or active fragment thereof and delivering the treated stent body, vascular graft or implant percutaneously or transluminally into a body lumen.

Preferably the body lumen is a vascular lumen.

The method of the fourth aspect may include treatment of the stent body, vascular graft, implant or the like with a heparin binding growth factor or active fragment thereof and/or a source of cell adhesive protein or active
5 fragment thereof.

Preferably, the method of the fourth aspect includes treatment of the stent body or vascular graft implant or the like with a heparin binding growth factor or active fragment thereof, and optionally a source of cell adhesive protein or active fragment thereof.

10 Preferably the body lumen is a vascular lumen.

In a fifth aspect, the present invention provides a surgical device such as a stent, vascular graft, implant or the like treated on at least a portion thereof with perlecan or active fragment thereof.

The device may further be treated with a heparin binding growth
15 factor or active fragment thereof and/or a source of cell adhesive protein or active fragment thereof.

Preferably, the device has been further treated with a heparin binding growth factor or active fragment thereof, and optionally a source of cell adhesive protein or active fragment thereof.

20 The device may be formed from any suitable material. The device may be formed from a suitable metal, ceramic or polymer. The device may be formed from a biomaterial.

The perlecan or active fragment thereof, and/or cell adhesive protein or active fragment thereof, may be coated on, or chemically bound to, a
25 surface of the device. The heparin binding growth factor is bound to the surface by its interaction with perlecan or an active fragment thereof.

Where plasma or serum is used as the source of cell adhesive protein, the device may be exposed to the plasma or serum.

The source of cell adhesive protein may be plasma or serum.
30 Preferably the plasma or serum is of human origin. The plasma or serum may originate from the treated subject at the time of the procedure.

In order that the present invention may be more readily understood, we provide the following non-limiting examples.

Brief Description of the Drawings

Figure 1 shows Western blots and SDS -PAGE(1a) and COA-
5 PAGE(1b) of purified human endothelial perlecan;

Figure 2 is a graph showing adhesion of human arterial smooth muscle cells (HASMC) and human umbilical arterial endothelial cells (HUAEC) in response to an increasing concentration of purified perlecan;

Figure 3 is a graph showing cell adhesion of HASMC cells or HUAEC
10 cells to either intact perlecan or the protein core of perlecan. Fibronectin is shown as the control;

Figure 4 is a graph showing the binding affinity between bFGF and surface coated perlecan;

Figure 5 shows growth of HUAEC cells (5a) and of HASMC cells
15 (5b) in the presence of bFGF and combinations thereof with perlecan and fibronectin fetal calf serum. The cells are grown in 10% fetal calf serum which has been depleted of fibronectin, vitronectin and heparin binding growth factor;

Figure 6 shows HUAEC cell growth on a perlecan coated surface which has
20 been exposed to plasma with bFGF in the growth medium (6A), HUAEC cell growth on a perlecan coated surface which has been exposed to plasma with surface bound bFGF only (6B), HUAEC cell growth on a perlecan coated surface which has been exposed to plasma with vascular endothelial cell growth factor (VEGF) in the medium (6C), and HUAEC cell growth on a perlecan coated surface which has been exposed to
25 plasma with surface bound VEGF (6D);

Figure 7 is a graph showing relative platelet attachment to different coating treatment compositions as shown.

EXPERIMENTAL PROCEDURES

MATERIALS

Heparin (H3149), Tris, Triton X-100, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), were purchased from Sigma.

Heparitinase I (heparinase III) was from Seikagaku Corporation Co. All other chemicals were of analytical grade. Tissue culture plastic ware was from either Nunc or Corning. Medium 199 containing Earles salts was from Gibco. All solutions for endothelial cell culture were prepared using pyrogen free water (Baxters) and small aliquots were filtered through sterile Zetapore 0.2 μm pore nylon membranes (Cuno Pacific). All re-useable glassware was treated with E-Toxa clean (Sigma) to remove pyrogens, and following several washes in tap and distilled water, was rinsed in pyrogen free water before sterilisation by autoclaving at 120 $^{\circ}\text{C}$ for 1 h followed by dry heat at 170 $^{\circ}\text{C}$ for 3 h. All handling of materials was done with surgical gloves. Pre-cast 4-15% polyacrylamide gels, pre-stained protein standards, the Protean II electrophoresis, blotting system and Coomassie Blue R250 were from Bio-Rad. Recombinant [^{125}I]labeled bFGF, peroxidase conjugated streptavidin and biotin conjugated rabbit anti-mouse IgG were purchased from Amersham. Peroxidase conjugated to rabbit anti mouse IgG, unconjugated rabbit anti-mouse IgG and alkaline phosphatase conjugated rabbit anti-mouse IgG were purchased from Dako. Bromochloroindolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate for alkaline phosphatase was purchased from Promega. Cn-Br activated Sepharose 4B, heparin Sepharose, DEAE Sepharose, Protein A-Sepharose and Gelatin Sepharose were from AMRAD-Pharmacia Biotech. Foetal calf serum was from P.A. Biologicals. Anti-Heparin antibody 10E4 was purchased from Seikagaku Corporation Co. Depleted sera was prepared according to the method of Underwood and Steele, (1991).

CELL CULTURE

Primary cultures of human umbilical arterial endothelial cells (HUAECs) were prepared from fresh umbilical cords delivered by caesarean section at Royal North Shore Hospital, Sydney, using 0.1% bacterial collagenase (Weis et al., 1991). Cells were grown on tissue culture plastic precoated for 2h at 37°C with bovine fibronectin (10 µg/ml), purified from fresh plasma by affinity chromatography on gelatin-Sepharose as described (Ruoslahti et al., 1982). Culture medium was Medium 199 with Earle's Salts containing 20% pyrogen free foetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 100 µg/ml heparin and 2% bovine brain extract prepared as described (Maciag et al., (1979)). Cells were passaged at a 1:3 split ratio after disaggregation with 0.125% trypsin, 0.02% EDTA, and used between passages 6 and 10. Conditioned medium was prepared by culturing endothelial cells in complete medium for up to 72 h. The medium was collected, centrifuged at 2000 g to remove cellular debris and stored at -20°C.

The C11STH cell line is derived from a spontaneously transformed human umbilical venous endothelial cell culture and has been characterised elsewhere (Cockerill et al., 1994). These cells were grown under the same conditions that were used for the HUAECs.

HASMCs were cultured by explant outgrowth from pieces of internal mammary arteries discarded at surgery as described previously (Whitelock et al., 1997). Smooth muscle cells were maintained in medium 199 containing 20% foetal calf serum (FCS), 4mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were passaged at a 1:2 or 1:3 split ratio after disaggregation with 0.125% trypsin 0.02% EDTA, and used between passage 4 and 6.

All cultures were checked for cell type by immunofluorescence using a monoclonal antibody specific for smooth muscle α actin (Sigma) or a polyclonal anti-factor VIII antibody (Dako).

ANTI-PERLECAN ANTIBODIES

The anti-perlecan monoclonal antibodies were the result of a fusion using Sp2/0 myeloma cells and spleen cells from mice immunized with whole bovine corneal endothelial cell ECM, described previously (Underwood and Bennett, 1989). The specificity of these antibodies has been described elsewhere including identification of perlecan domain specificity for some of them (Whitelock et al., 1996). Monoclonal antibodies A 71, A74, and A76 were purified from ascites fluid by affinity chromatography on protein A-Sepharose.

PURIFICATION AND CHARACTERISATION OF HUMAN PERLECAN

Conditioned medium from confluent cultures of human endothelial cells was batched (approx. 2 litres) and passed over (1 ml/min) a DEAE Sepharose column (100ml bed volume) which had been equilibrated with 20mM Tris, 250mM NaCl, 10mM EDTA, 1 mM PMSF, 1 mM benzamidine, pH 7.5. The column was washed with the start buffer until a baseline was achieved and the bound proteins containing the perlecan were eluted with a step gradient of 1M NaCl in 20mM Tris, 10mM EDTA, 1 mM PMSF, 1 mM benzamidine, pH 7.5. The peak from the 1M NaCl elution was then passed over an immunoaffinity column of A71 antibodies conjugated to CnBr activated Sepharose 4B (5mg A71 per ml of Sepharose 4B; 5 ml bed volume). The eluate was cycled over the column for four - five hours at a flow rate of approx. 1 ml/min. The column was washed with 20mM Tris, 10mM EDTA, 1M NaCl, 1 mM PMSF, 1 mM benzamidine, pH 7.5 to achieve a baseline and the bound perlecan was eluted with 6M urea in PBS. This fraction was then diafiltered against PBS in Centricon 10 or 30 concentrating vessels (Amicon Industries) to remove any traces of urea. The presence of perlecan core protein and heparan sulfate were monitored in column fractions using antibodies to either the protein core or HS in an ELISA. ELISAs were performed as described previously (Underwood et al., 1992) using biotin conjugated rabbit anti-mouse IgG and peroxidase conjugated to streptavidin

to enhance the signal. ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate reaction absorbance was read at 405 nm in a Bio-Rad 3550 plate reader. The reference wavelength was 490 nm. The purified perlecan was then quantitated using a micro-BCA assay (Pierce Industries) and stored
5 at -70°C until used.

SDS-PAGE AND COA-PAGE ELECTROPHORESIS AND IMMUNOBLOTTING

SDS-PAGE was performed using 4-15 % polyacrylamide gradient gels as described by Laemmli (1970). Composite agarose- polyacrylamide gel
10 electrophoresis (COA-PAGE) was performed as described by McDevitt and Muir (1971) except that the gels did not contain β -dimethyl aminopropionitrile. After electrophoresis some of the SDS-PAGE gels were stained with 0.25% w/v Coomassie Blue R250 in 45% v/v methanol, 10% v/v acetic acid in H₂O for 30 minutes at room temperature, and destained in the
15 same buffer. Some SDS-PAGE and COA-PAGE gels were transferred to nitrocellulose or PVDF membranes (Towbin et al., 1979) and probed with the antibodies directed against either the protein core or heparan sulfate of perlecan. Immunoreactive bands were detected using alkaline phosphatase conjugated rabbit anti-mouse IgG as secondary antibody and
20 bromochloroindolyl phosphate (BCIP) with nitro blue tetrazolium (NBT) as substrate.

Example 1

25 *CELL ADHESION AND GROWTH STUDIES*

The wells of a 96 well tissue culture plate were coated with either fibronectin (10 μ g/ml), perlecan at the concentrations indicated, or a mix of the two for 2 hours at 37°C plus 16 hours at 4°C. To remove the heparan sulfate from the proteoglycan, some wells were incubated with 0.05 U/ml
30 heparitinase I diluted in 1% BSA (bovine serum albumin) in PBS for 2 hours

at 37° C. The plates were washed twice with 1% BSA in PBS prior to the addition of the cells or analysis by ELISA. Cell adhesion studies were performed by plating either HASMCs or HUAECs at 2×10^4 cells/well and incubating the cultures for two hours in the presence of medium 119
5 containing either 1% BSA or 10% serum which had been depleted of both vitronectin and fibronectin (double depleted serum; DDS) (Underwood and Steele, 1991). Non-adherent cells were washed off and the cells adhering to the wells were fixed with 10% TCA, and stained with crystal violet as described previously (Kueng et al., 1989). The stain was solubilised in 10%
10 acetic acid (100 μ l/ well) and quantitated by measuring the absorbance of the wells at 595nm using 405nm as the reference wavelength. Cell growth studies were performed over six days in wells which had been coated with either fibronectin or perlecan as described above and which had subsequently been incubated with 100ng/ml bFGF (in 1% BSA/PBS) for 2
15 hours at 37°C and washed prior to the addition of cells. For cell growth studies cells were plated at 3×10^3 cells/well and the cells were cultured for 6 days in medium 199 which had been supplemented with either 10% complete serum or 10% serum depleted of fibronectin, vitronectin, and heparin binding growth factor (triple depleted serum; TDS). To assess cell
20 growth, plates were fixed with either TCA or 4% formaldehyde in PBS on days one, three and six, stained with crystal violet and quantified as described above.

DETERMINATION OF DISSOCIATION CONSTANT; SCATCHARD ANALYSIS

25 The wells of a 96 well PVC microtitre plate were coated with 0.75 μ g/well of perlecan in a volume of 50 μ l for two hours at 37°C and then overnight at 4°C in a humidified atmosphere. The wells were blocked with 3% BSA in PBS for two hours at room temperature and then rinsed with PBS. The perlecan coated wells were incubated with an increasing concentration
30 of unlabelled bFGF in the presence of 35 fmoles/well of 125 I-labeled bFGF

(approx. 40000 CPM/well) until equilibrium was reached (2 hours at room temperature). First, the proportion, x of ^{125}I -labeled bFGF which could bind to perlecan was calculated using the equation $x = 1 - A_2/A_1$ where A_1 and A_2 are the bound counts obtained by two sequential exhaustive incubations
5 of the same solution of ^{125}I -labeled bFGF (in absence of unlabeled bFGF) on perlecan coated wells. Secondly, $\alpha/(1 - \alpha)$ was plotted against αL where α is the proportion of bindable ^{125}I -labeled bFGF bound to the wells (CPM) and L is the molar concentration of unlabeled bFGF. The slope of the straight line obtained is equal to $-1/K$ where K is the dissociation constant and the
10 intercept on the y axis is P/K where P is the molar concentration of bFGF binding sites on perlecan bound to the well.

RESULTS

15 *PURIFICATION AND CHARACTERISATION OF HUMAN PERLECAN*

Human perlecan was purified from the conditioned medium of endothelial cells by sequential DEAE Sepharose and A71 Sepharose immunoaffinity chromatography. The presence of perlecan core protein along with the presence of HS was monitored throughout the purification
20 schedule by an ELISA using A76 and 10E4 antibodies, respectively. The perlecan from the conditioned medium bound the DEAE column and was released by washing the column with 1M NaCl. If sequential fractions were taken throughout the wash, the perlecan eluted later suggesting that it was released from the column with a relatively high concentration of NaCl (data
25 not shown). The peak containing the perlecan was applied directly to the immunoaffinity column and eluted with urea. When samples from this peak were run on 4 - 15% SDS-PAGE gels, a single band was demonstrated with either Coomassie blue, alcian blue or silver staining. This band was mainly at the bottom of the loading well but on some occasions it smeared into the
30 running gel (Fig. 1a). When these were blotted to PVDF and probed with

either the anti-protein core antibody (A76) or the anti-HS antibody (10E4) the same band at the top of the running gel was reactive with the antibodies (Fig. 1a). The smeared nature of the perlecan band was more evident in the Western blots than in the gels. Other purified samples were electrophoresed through composite agarose/acrylamide gels (COA-PAGE)(Fig. 1b). Molecules analysed in this system migrated according to the ratio of their size to charge. The intact proteoglycan (with HS) migrated approximately 30 mm into the gel under the conditions used. The smeared nature of the band was more evident in the lane stained with 10E4 than with A76 (control lanes in Fig. 1b). When the HS was removed with heparitinase, the bulk of the negative charge was removed leaving the protein core which migrated approximately half the distance into the gel. The naked protein core was still reactive with A76 but as expected there was no reactivity with 10E4 (Fig.1b).

Fractions containing purified perlecan were screened in an ELISA for the presence of other ECM molecules and found to be free of contaminating fibronectin, vitronectin, laminin, collagen types I, III, IV and V, versican, decorin, CS and elastin (data not shown).

VASCULAR CELL ADHESION TO PERLECAN

Perlecan was adsorbed onto the wells of a tissue culture plate (polystyrene) and either human endothelial (HUAEC) or smooth muscle cells (HASMC) were plated onto it in the absence of the serum proteins, fibronectin and vitronectin. The number of cells attached to perlecan coated wells was compared to that which attached to wells coated with fibronectin. At a coating concentration of 20 µg/ml, both cell types adhered to perlecan with the HASMCs adhering better (80%) than the HUAECs (35%) (Fig.2). The cells attached to perlecan at 2 hours were not as well spread as those cells plated onto fibronectin. By 16 hours, the cells plated on perlecan were spread further but still had refractile edges compared to those cells on fibronectin. This suggested they were still not as well spaced as the cells

coated on fibronectin (data not shown). The role of HS in the attachment of cells was investigated by incubating perlecan coated wells with heparitinase which removed the glycosaminoglycan. Cells attached slightly better to the protein core than to the intact proteoglycan with an average increase of
5 approximately 10% when the HS was removed (Fig. 3). The cells also were better spaced when attached to heparitinase treated perlecan. Perlecan purified from primary endothelial cells was less effective at attaching cells to polystyrene than that purified from the endothelial cell line (C11STH). When the HS was removed, however, the amount of cell attachment to either
10 protein core was almost identical. Incubation of the cells with 100 µg/ml of antibodies against domain I during the assay had no effect on the attachment of cells to the protein core.

DISSOCIATION CONSTANT BETWEEN PERLECAN AND bFGF

15 The dissociation constant between perlecan coated onto a surface and bFGF in solution was quantitated at 50nM (Fig.4). From the Scatchard plot, the molar concentration of perlecan in each well was obtained from the formula $P/K = y\text{-intercept}$. The y-intercept of the graph in Fig. 4 was equal to 0.95. Therefore, $P = 4.75 \times 10^{-8}$ M which equals 30.4 µg/ml. If we assume
20 there are 3 HS chains on each perlecan molecule and one bFGF molecule binds per chain, we get a value of 0.5 µg of active perlecan bound to each well.

THE PRESENTATION OF BOUND bFGF BY PERLECAN

25 To test the biological significance of the interaction between perlecan and the growth factor, cells were grown on a surface which had been coated with perlecan, fibronectin or a mix of the two and which had been saturated with bFGF. The culture medium in these experiments did not include any added growth factor, and was supplemented with 10%
30 whole serum or TDS. For all the experiments described, the concentration

of perlecan used for coating was 10 µg/ml. This was chosen because it gave a reasonable amount of cell adhesion on its own (Fig. 2) and did not affect the cell attachment to fibronectin. Also, an ELISA using antibodies against either perlecan (A76), HS (10E4) or fibronectin (A17) demonstrated that

5 when wells were coated with the mix of the two molecules (i.e. perlecan at 10 µg/ml and fibronectin at 10 µg/ml), the amount of immunoreactivity seen was the same as when they were coated separately. This suggested that the two molecules were present at similar concentrations in the wells incubated with the mix of fibronectin and perlecan and that they were not competing

10 with each other for absorption to the polymer surface. The presence of fibronectin did not affect the reactivity of the anti-HS antibody suggesting that the fibronectin was not binding to the HS and masking its reactivity. Cells plated on wells coated with a mix of perlecan and fibronectin grew better than on other surfaces which did not have perlecan in the coating mix

15 (Fig. 5a, b). HUAECs plated into wells which had been incubated with bFGF in the absence of either perlecan or fibronectin had died by day 6 (Fig. 5a). HASMCs grown in the presence of serum which had been stripped of fibronectin, vitronectin and heparin binding growth factors, grew better on the surfaces modified with both perlecan and fibronectin than on surfaces

20 modified with fibronectin or those surfaces which were unmodified (Fig 5b). The growth promoting activity of perlecan in the coating mixture was not replaced by the addition of heparin suggesting that the protein core of perlecan was required to anchor the proteoglycan to the surface.

Perlecan was shown to bind bFGF with a Kd of approx 50 nM which

25 is similar to previously published dissociation constants for the growth factor and perlecan. The aim of these studies was to assess the functional role of perlecan in the growth of vascular cells in response to bound bFGF. Both human arterial smooth muscle cells (HASMC) and endothelial cells (HUAEC) adhered to perlecan coated on tissue culture polystyrene at about

30 80% and 35%, respectively when compared to the level of cell attachment to fibronectin. When HUAECs were grown on a coating of both fibronectin and

perlecan which had been pre-incubated with bFGF, the cells grew for up to six days in absence of any added growth factors in the medium. If perlecan was absent, the cells did not grow and there were no viable cells present in the wells at day three. Therefore, these results demonstrate a role for
5 perlecan in the proliferation of endothelial cells in response to vascular injury. They also suggest that perlecan may have a wider role in the response of other cell types to bFGF and in particular an integral role in the wound healing situation.

10 **Example 2**

METHODS

CELL PROLIFERATION ASSAYS

15 The wells of a 96-well plate were coated with either fibronectin (10 $\mu\text{g/ml}$) or perlecan (10 $\mu\text{g/ml}$) for 2 hours at 37°C, followed by 16 hours at 4°C. The growth factor of choice (either bFGF or VEGF) were added to the wells at a concentration of 100 ng/ml diluted in medium 199 containing 10% human plasma followed by incubation for 4 h at 37°C in a humidified atmosphere
20 containing 5% CO₂. Wells were washed two times with either medium 199 or phosphate buffered saline. Endothelial cells (HUAEC) were added to the wells (3 x 10³ cells/well) in medium 199 containing 10% serum depleted of fibronectin and vitronectin. In some treatments, the growth factor was omitted from the plasma treatment, but was included in the growth medium.
25 At days 1, 3 and 6, an MTS assay (Promega) was performed which gives a measure of cell proliferation.

PLATELET ADHESION ASSAYS

Sample wells were pre-coated with purified proteins, human plasma or PBS
30 using the same conditions as described above for the proliferation assays.

Platelets were harvested from human blood collected from healthy donors by venous phlebotomy. Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 187 g for 20 minutes at room temperature followed by careful removal of the upper platelet-rich layer. To separate
5 platelets from plasma proteins, PRP was centrifuged at 500 g for 15 minutes at room temperature. After washing the pellet with tyrodes buffer they were counted in a haemocytometer and the concentration adjusted to 1×10^8 /ml in tyrodes plus 0.1 U/ml apyrase. Immediately prior to platelet adhesion, both Ca^{2+} and Mg^{2+} were added to the platelet suspension to a final
10 concentration 1 mM. The wells were aspirated and 1×10^8 platelets/ml added to each well ($100 \mu\text{l}$ /well) and allowed to settle for 45 minutes at 37°C . The wells were then rinsed 8 times with PBS, and platelet adhesion was determined by a LDH (Lactate Dehydrogenase) method. The adhered platelets were lysed by adding $200 \mu\text{l}$ /well 1% Triton X-100 at 37°C for 30
15 minutes. $100 \mu\text{l}$ of lysate was mixed with an equal volume of LDH substrate (Cytotoxicity Detection Kit (LDH) Catalog Number 1-644-793, Boehringer Mannheim, Germany) and then incubated at 37°C for 30 minutes. The absorbance was measured at 490 nm against a 650 nm reference using a BioRad 3550 (BioRad, UK) microplate reader.

20

Results

CELL PROLIFERATION ASSAYS

In the previous example, a cell adhesive protein was used in
25 combination with perlecan to obtain cell growth (see figs 5A and 5B). In this example, the extra cell adhesive protein is provided by exposing the surface to human plasma. This step is performed in conjunction with binding the heparin binding growth factor. This is the methodology used to generate the data shown in Figs 6A, B, C and D.

Endothelial cell growth on a perlecan coated surface which has been exposed to plasma, with basic fibroblast growth factor (bFGF) in the culture medium is shown in Fig. 6A. Endothelial cell growth on a perlecan coated surface which has been exposed to plasma, with surface bound bFGF only is
5 shown in Fig 6B.

Endothelial cell growth on a perlecan coated surface that has been exposed to plasma, with vascular endothelial cell growth factor (VEGF) in the medium is shown in Fig. 6C. Endothelial cell growth on a perlecan coated surface which has been exposed to plasma, with surface bound VEGF
10 only is shown in Fig 6D.

PLATELET ADHESION ASSAYS

A perlecan coated surface was compared to a non-adhesive surface (albumin coated) and an adhesive surface (plasma coated) for its ability to adhere
15 platelets. These results demonstrate that perlecan does not adhere platelets (Fig. 7). Furthermore, we have demonstrated that when a perlecan coated surface is exposed to plasma, it reduces the amount of platelets which adhere to the plasma proteins.

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15 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A wound or injury treatment composition including perlecan or active fragment thereof and a pharmaceutically acceptable carrier or diluent.
- 5 2. A wound or injury treatment composition according to claim 1 further including a heparin binding growth factor or active fragment thereof, and/or a cell adhesive protein or active fragment thereof.
3. A wound or injury treatment composition according to claim 1 wherein the composition includes a cell adhesive protein or active
10 fragment thereof.
4. A composition according to any one of claims 1 to 3 wherein the perlecan is human perlecan.
5. A composition according to any one of the preceding claims wherein the cell adhesive protein is selected from fibronectin or vitronectin or
15 a combination thereof.
6. A composition according to any one of the preceding claims wherein the heparin binding growth factor is selected from the group consisting of basic fibroblast growth factor (bFGF), transforming growth factor α , transforming growth factor β , epidermal growth factor, platelet derived growth factor, heparin binding epithelial
20 growth factor, granulocyte colony stimulating factor, vascular endothelial growth factor (VEGF), VEGF-related growth factor, acidic FGF and other members of the FGF family.
7. A composition according to claim 6 wherein the heparin binding growth factor is bFGF or VEGF.
25
8. A composition according to any one of the preceding claims further including at least one wound healing agent or an agent for treatment of symptoms accompanying wounds.

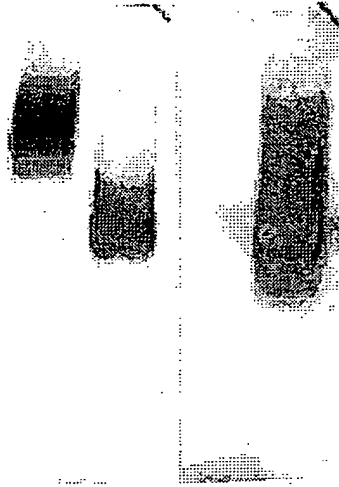
9. A composition according to claim 8 wherein the at least one further agent is selected from the group consisting of anti-bacterial agents, anti-viral agents, anti-fungal agents and local anaesthetics.
- 5 10. A composition according to any one of the preceding claims in the form of a liquid, semi-solid or solid.
11. A composition according to any one of the preceding claims in a form providing controlled or sustained release.
12. A composition in accordance with any one of the preceding claims wherein the carrier in the form of a bandage.
- 10 13. A composition according to any one of the preceding claims wherein the wound or injury is in the form of cellular damage resulting from a burn, sore, incision, wound, vascular injury or trauma occasioned by a surgical procedure.
- 15 14. A method for the treatment of an injury in a subject, the method including administering to the subject perlecan or an active fragment thereof.
15. A method according to claim 14 wherein the treatment includes administration of a heparin binding growth factor or active fragment thereof and/or a source of a cell adhesive protein.
- 20 16. A method according to claim 14 wherein the method includes administration of a heparin binding growth factor and optionally a cell adhesive protein.
17. A method according to any one of claims 14 to 16 wherein the injury is in the form of cellular damage resulting from a burn, sore, incision
25 or wound or cellular injury or trauma occasioned by a surgical procedure.
18. A method for inhibiting or reducing restenosis and/or thrombosis in a subject, the method including treating the subject with perlecan or active fragment thereof.

19. A method according to claim 18 which includes treatment with a heparin binding growth factor or active fragment thereof, and/or a source of cell adhesive protein or active fragment thereof.
20. A method according to claim 17, 18 or 19 wherein the treatment takes place before, during or following coronary angioplasty, atherectomy, arterial bypass graft surgery, biomaterial implantation or the like.
21. A method for preventing or reducing restenosis and/or thrombosis in a body lumen treated to open a luminal restriction, the method including the steps of providing a surgical device selected from a stent body, vascular graft, implant or the like, treating the device with perlecan or active fragment thereof and delivering the treated device percutaneously or transluminally into a body lumen.
22. A method according to claim 21 wherein device is further treated with a heparin binding growth factor or active fragment thereof or a source of cell adhesive protein or active fragment thereof.
23. A method according to claim 21 wherein device is further treated with a heparin binding growth factor or active fragment thereof and optionally a source of cell adhesive protein or active fragment thereof.
24. A surgical device selected from a stent body, vascular graft, implant or the like at least a portion of which is treated with perlecan or active fragment thereof.
25. A device according to claim 24 which is further treated with a heparin binding growth factor or active fragment thereof or a source of cell adhesive protein or active fragment thereof.
26. A device according to claim 24 which is further treated with a heparin growth factor and optionally a source of cell adhesive protein.
27. A device according to any one claims 24 to 26 which is formed from a biocompatible metal, polymer or ceramic.
28. A method according to any one of claims 15 to 17, 19, 20, 22 or 23, or a device according to any one of claims 25 to 27, wherein the heparin binding growth factor is selected from the group consisting of basic

- fibroblast growth factor (bFGF), transforming growth factor α , transforming growth factor β , epidermal growth factor, platelet derived growth factor, heparin binding epithelial growth factor, granulocyte colony stimulating factor, vascular endothelial growth factor (VEGF), VEGF-related growth factor, acidic FGF and other members of the FGF family.
- 5
29. A method according to claim 28 wherein the heparin binding growth factor is bFGF or VEGF.
30. A method according to any one of claims 15 to 17, 19, 20, 22 or 23, or
10 a device according to any one of claims 25 to 27 wherein the cell adhesive protein is selected from fibronectin or vitronectin or a combination thereof.
31. A method according to any one of claims 15 to 17, 19, 20, 22 or 23, or
15 a device according to any one of claims 25 to 27 wherein the source of cell adhesive protein is plasma or serum.
32. A method or device according to 31 wherein the plasma or serum is of human origin.
33. A method or device according to claim 31 or 32 wherein the serum or plasma originates from the treated subject at the time of treatment.

Western blot of COA-PAGE

h'ase control h'ase control



A76

10E4

1b

SDS-PAGE



Western blot



- 208
- 144
- 87
- 44.1
- 32.7

A76 10E4

**Mr
stds**

1a

Figure 1

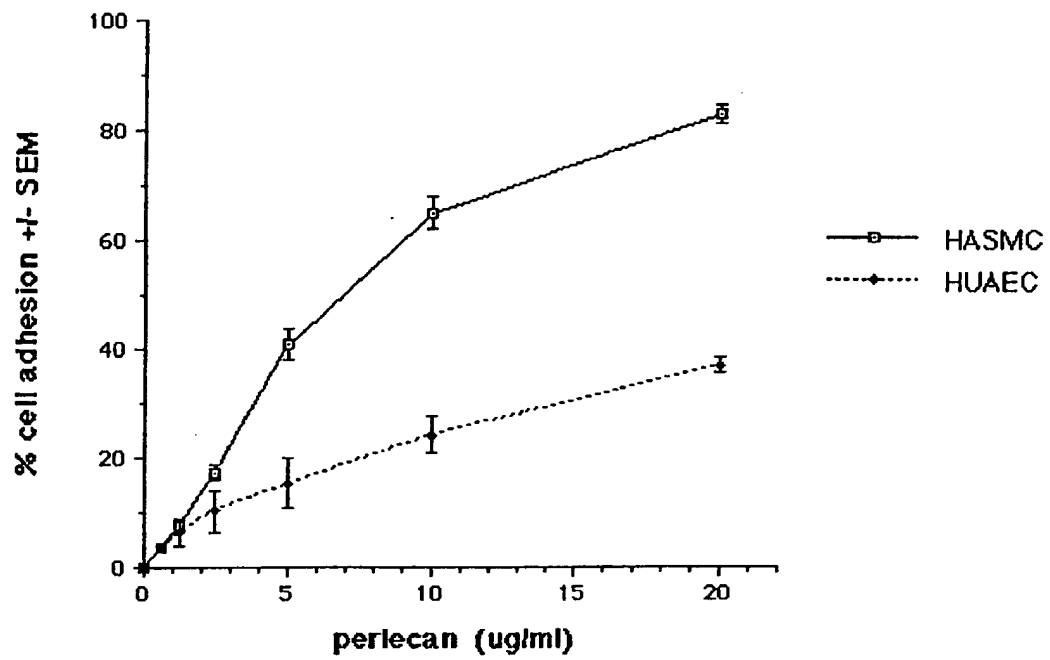


Figure 2

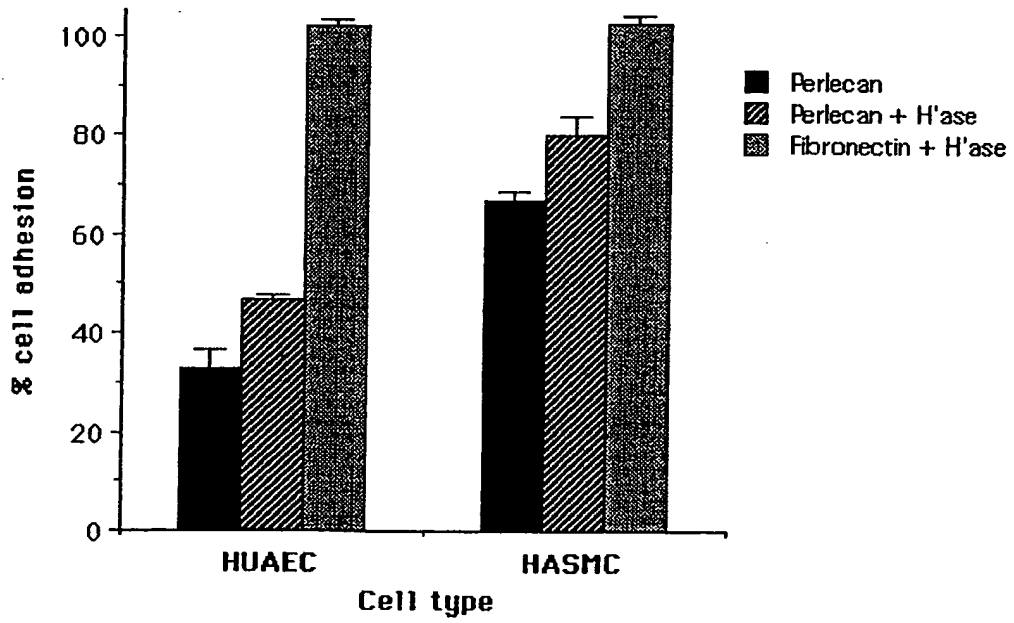


Figure 3

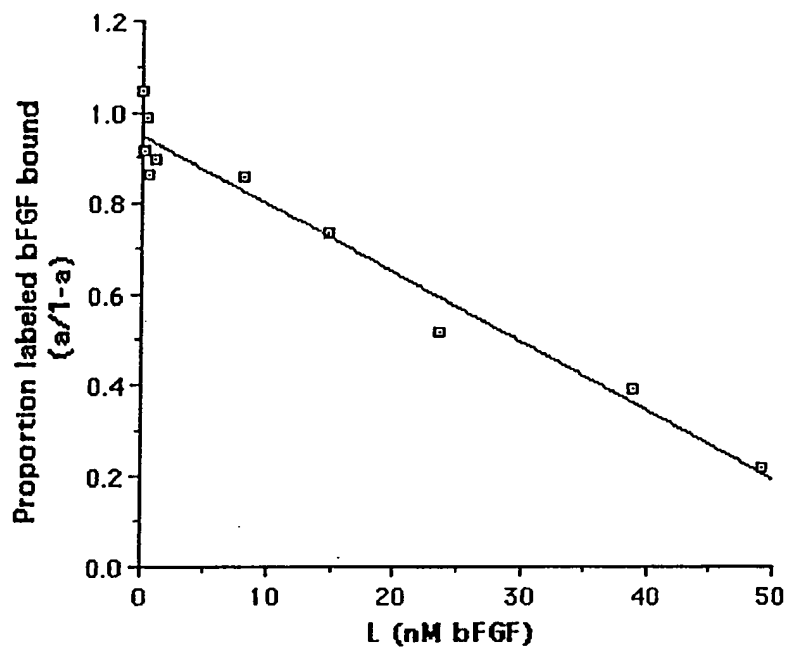


Figure 4

Fig 5A

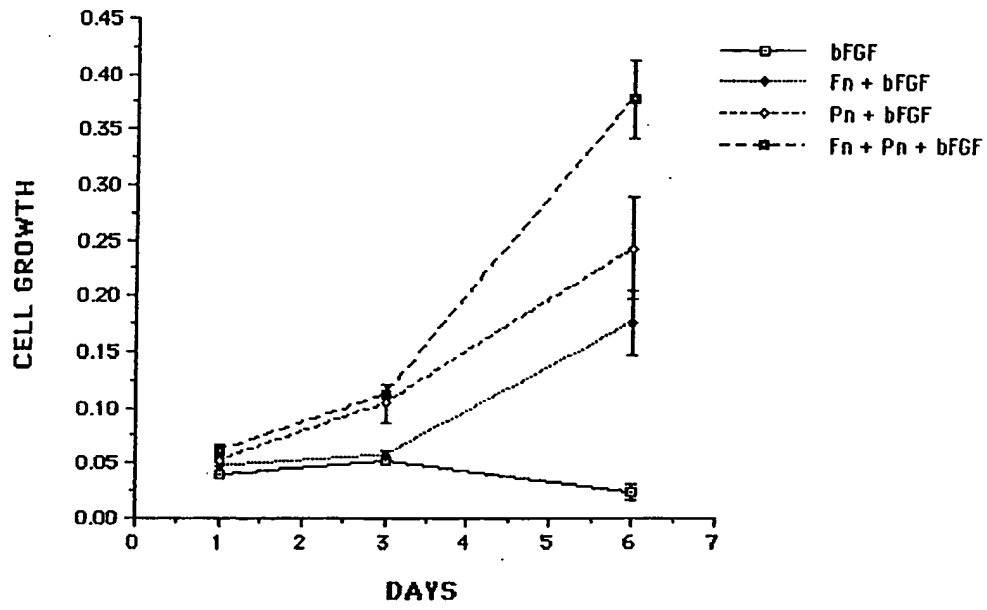
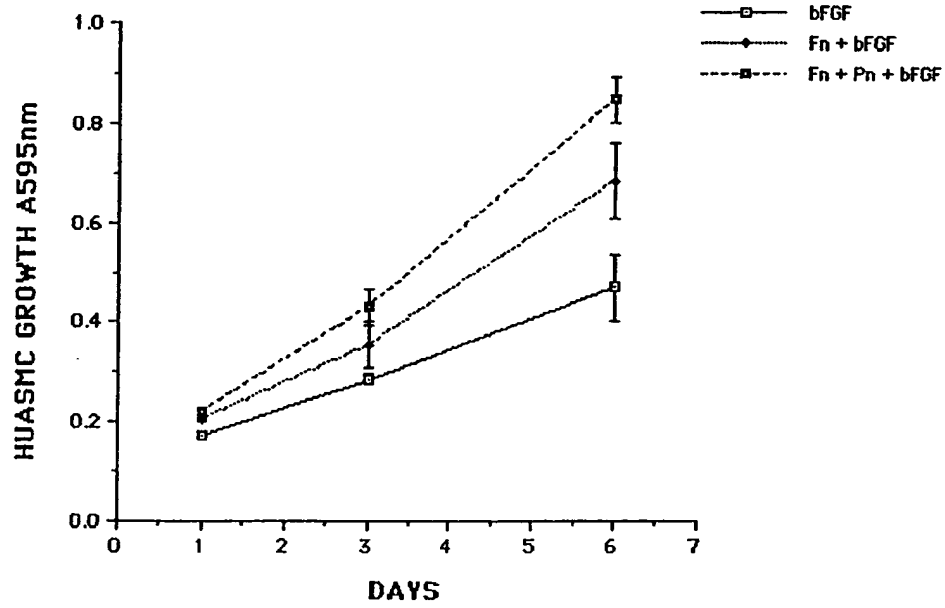
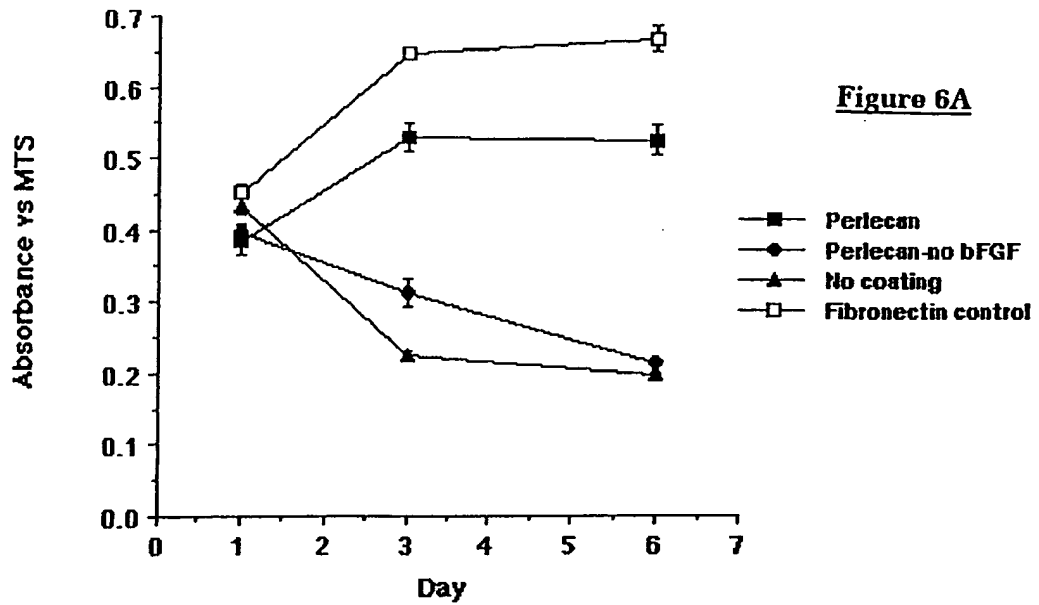


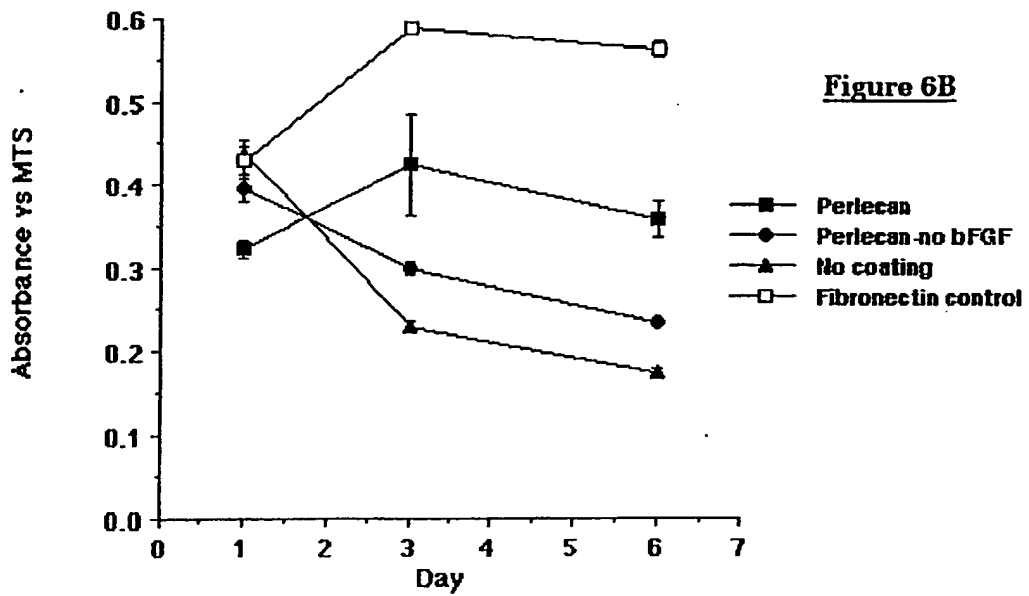
Fig 5B



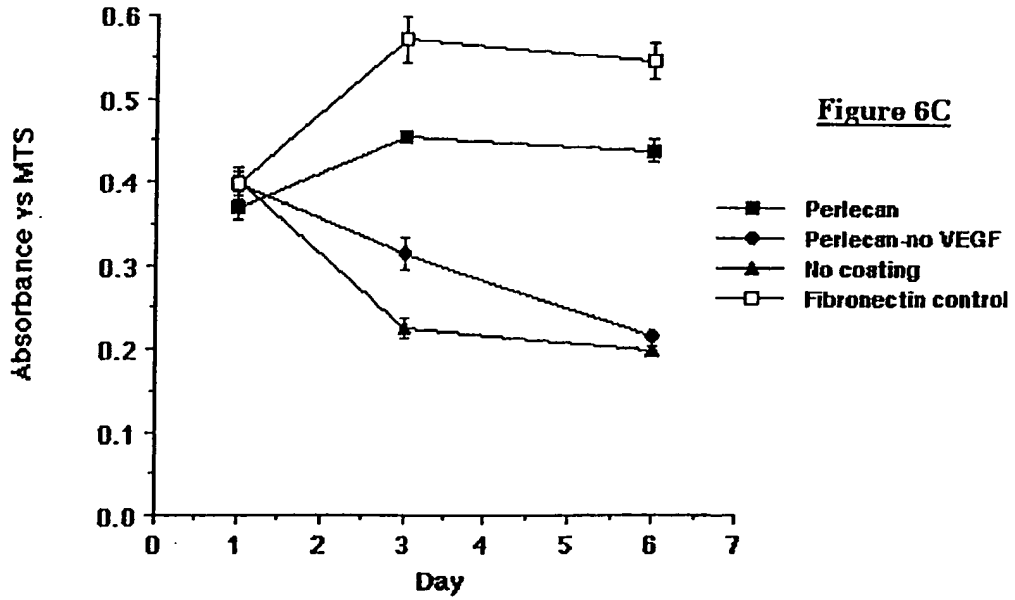
Proliferation of HUAECs - bFGF in solution.



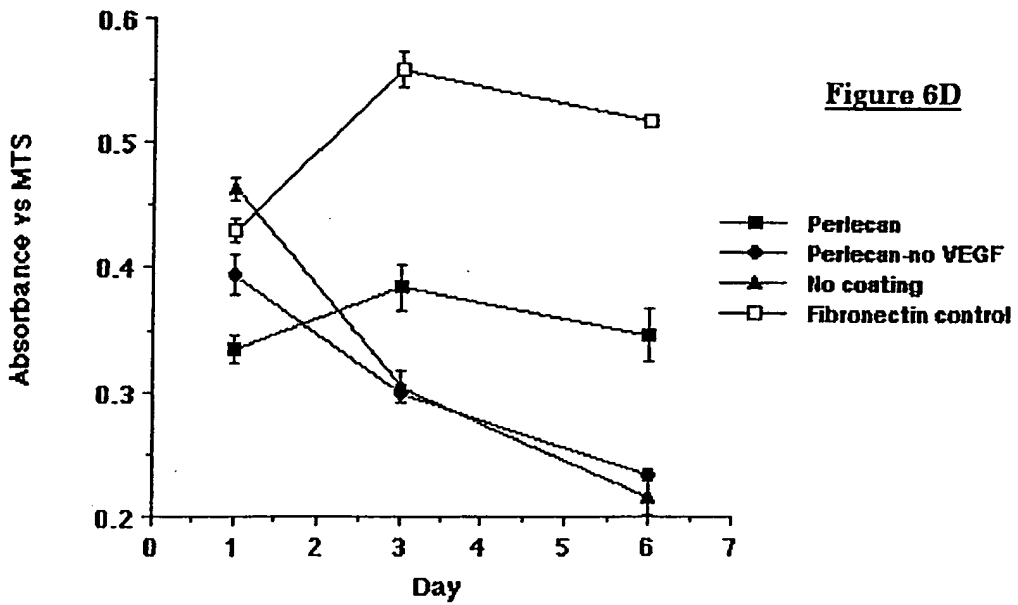
Proliferation of HUAECs - bFGF on surface.



Proliferation of HUAECs - VEGF in solution.



Proliferation of HUAECs- VEGF on surface.



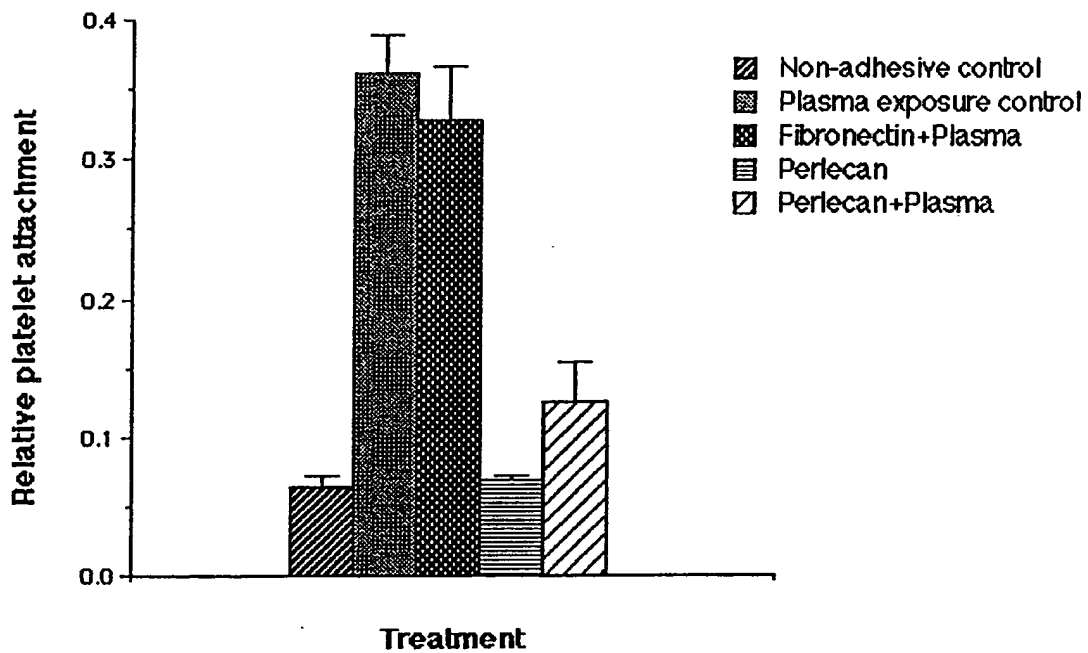


Figure 7

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 98/00608

A. CLASSIFICATION OF SUBJECT MATTER		
Int Cl ⁶ : A61K 31/725, 31/73; 38/18; 38/19; 38/30; 38/39; A61F 2/06; A61L 27/00, 33/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC : see keywords below		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DERWENT : Heparin: Proteoglycan.; Heparan: Proteoglycan.; HSPG, perlecan MEDLINE, CHEMICAL ABSTRACTS : Perlecan, Wound Heal?, wound, antithrombo? anticoagulant?, Prosthe?, basic fibroblast growth factor		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	J. of Biol. Chem. (1996) Vol 271 (17) pages 10079-10086 "The Degradation of Human Endothelial Cell-derived Perlecan and Release of bound Basic Fibroblast Growth Factor by Stromelysin, Collagenase, Plasmin, and Heparanases" J.M. Whitelock et al. whole document, abstract	1-33
X,Y	Cell (1994) Vol 79 pages 1005-1013 "Perlecan, Basal Lamina Proteoglycan, Promotes Basic Fibroblast Growth Factor-Receptor Binding, Mitogenesis, and Angiogenesis" D. Aviezer et al. whole document, abstract	1-33
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search 24 August 1998		Date of mailing of the international search report 21 SEP 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer JAYNE BRITON Telephone No.: (02) 6283 2246

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 98/00608

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93/05167 (CHILDREN'S MEDICAL CENTER CORPORATION) 18 March 1993 whole document (for example, page 4 lines 5 to 14; page 17 lines 6 to 22)	1-33
Y	US 4963146 (SHU-TUNG LI) 16 October 1990 whole document (for example, column 9 lines 14 to 19; column 10 line 39 to 47; column 16 lines 27 to 33)	1-33
Y	US 5206023 (E.B. HUNZIKER) 27 April 1993 whole document (for example, column 3 line 34 to 60; column 4, lines 31 to 36; and lines 49 to 63)	1-33
P,X	AU 42362/97 (UNIVERSITY OF WASHINGTON) 5 March 1998 whole document; page 80 to line 12 to page 85 line 10; claim 47	1-33
P,X	WO 98/24466 (ALCON LABORATORIES, INC) 11 June 1998 whole document, claim 9	1-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 98/00608

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	93/05167	AU	25617/92	US	5486599	WO	90/12033
		AU	71129/94	EP	705332	WO	95/00633
US	4963146	US	5026381				
US	5206023	AU	14128/92	CA	2101556	CN	1064813
		EP	569541	IL	100799	NO	932748
		NZ	260125	WO	92/13565	ZA	9200726
		US	5368858				
AU	42363/97	WO	98/08381				
END OF ANNEX							